Methods and Reagents For Modulating TGF-β Superfamily Signalling

Priority Statement

5   This application claims priority from United States provisional application 60/047,991, filed May 28, 1997.

Statement as to Federally Sponsored Research

This invention was made in part with support from the Federal government through NIH Grants Nos. HD24926 and HD29468. The Federal

10   government has certain rights in the invention.

Background of the Invention

TGF-β superfamily members signal through activation of transmembrane serine-threonine kinase receptors. These receptors phosphorylate and activate Smads, a novel class of signal transducers. Signals initiated by TGF-

15   β superfamily members are important for regulating cellular processes, including cell division, survival, differentiation, and specification of developmental fate throughout the growth and development of diverse organisms.

During early embryogenesis of the frog *Xenopus laevis*, the TGF-β growth factor family plays a central role in the specification and patterning of

20   various tissues: TGF-β superfamily members activin, Vg-1, and TGF-β all induce a full range of dorsal and ventral mesodermal markers in early embryonic tissue, whereas other TGF-β superfamily members specify axial pattern or epidermal, as opposed to neural, tissue. Almost all the critical patterning events in early *Xenopus* embryogenesis appear to involve members of the TGF-β superfamily.

The transforming growth factor β (TGF-β) superfamily of cytokines, which includes bone morphogenic proteins (BMPs), activin, TGF-β, and Vg-1, regulate a wide range of normal and pathological biological processes. These processes include cell specification during development, terminal differentiation of

5   many cell types, fibrosis during wound healing or organ damage (e.g., cirrhosis), proliferation and invasiveness of normal and transformed cells, and angiogenesis and immune suppression induced by tumors (Roberts and Sporn, Peptide growth factors and their receptors I, eds. Sporn and Roberts, Berlin, Springer-Verlage, 419-473, 1990; Sporn et al., Science 33: 532-534, 1986). For example, one

10   member of the family, TGF-β, is secreted by a wide variety of tumors and has a wide variety of immunosuppressive effects, including the ability to induce apoptosis in B and T lymphocytes (Brabletz et al., Mol. Cell Biol. 13: 1155-1162, 1993; Cahouchi et al., Oncogene 11: 1615-1622, 1995; Weller et al., Exp. Cell Res. 221: 395-403, 1995). The ability to manipulate specific aspects of TGF-β

15   superfamily signalling *in vivo* would be a powerful tool both for understanding the role of these factors in normal embryonic patterning and for controlling a broad range of pathological processes.

<center>Summary of the Invention</center>

We have discovered methods and reagents for identifying compounds

20   that modulate TGF-β superfamily signalling. These methods and compounds are useful for the detection and treatment of conditions involving abnormal TGF-β superfamily signalling.

In the first four aspects, the invention provides methods for detecting compounds capable of modulating TGF-β superfamily signalling. The methods

25   include the steps of providing a cell having a reporter gene operably linked to a

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DNA-binding-protein recognition site, in addition to having either:

a) a first fusion gene capable of expressing a first fusion protein comprising a polypeptide fragment of Smad2 covalently bonded to a binding moiety capable of specifically binding to the DNA-binding-protein recognition site and a second fusion gene capable of expressing a second fusion protein comprising a polypeptide fragment of FAST-1 covalently bonded to a gene activating moiety,

b) a first fusion gene capable of expressing a first fusion protein comprising a polypeptide fragment of FAST-1 covalently bonded to a binding moiety capable of specifically binding to the DNA-binding-protein recognition site and a second fusion gene capable of expressing a second fusion protein comprising a polypeptide fragment of Smad2 covalently bonded to a gene activating moiety,

c) a first fusion gene capable of expressing a first fusion protein comprising a polypeptide fragment of Smad3 covalently bonded to a binding moiety capable of specifically binding to the DNA-binding-protein recognition site and a second fusion gene capable of expressing a second fusion protein comprising a polypeptide fragment of FAST-1 covalently bonded to a gene activating moiety, or

d) a first fusion gene capable of expressing a first fusion protein comprising a polypeptide fragment of FAST-1 covalently bonded to a binding moiety capable of specifically binding to the DNA-binding-protein recognition site and a second fusion gene capable of expressing a second fusion protein comprising a polypeptide fragment of Smad3 covalently bonded to a gene activating moiety; exposing the cell to the compound; and measuring reporter gene expression in the cell, where a change in the reporter gene expression indicates that the compound is capable of modulating TGF-β superfamily signalling.

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In the fifth, sixth, seventh, and eighth aspects, the invention features a cell useful for detecting a compound capable of modulating TGF-β superfamily signalling, the cell having a reporter gene operably linked to a DNA-binding-protein recognition site in addition to having either:

5   *a)* a first fusion gene capable of expressing a first fusion protein comprising a polypeptide fragment of Smad2 covalently bonded to a binding moiety capable of specifically binding to the DNA-binding-protein recognition site and a second fusion gene capable of expressing a second fusion protein, the second fusion protein comprising a polypeptide fragment of FAST-1 covalently bonded to

10   a gene activating moiety,

         b) a first fusion gene capable of expressing a first fusion protein comprising a polypeptide fragment of FAST-1 covalently bonded to a binding moiety capable of specifically binding to the DNA-binding-protein recognition site and a second fusion gene capable of expressing a second fusion protein, the second

15   fusion protein comprising a polypeptide fragment of Smad2 covalently bonded to a gene activating moiety,

         c) a first fusion gene capable of expressing a first fusion protein comprising a polypeptide fragment of Smad3 covalently bonded to a binding moiety capable of specifically binding to the DNA-binding-protein recognition site

20   and a second fusion gene capable of expressing a second fusion protein, the second fusion protein comprising a polypeptide fragment of FAST-1 covalently bonded to a gene activating moiety, or

         d) a first fusion gene capable of expressing a first fusion protein comprising a polypeptide fragment of FAST-1 covalently bonded to a binding

25   moiety capable of specifically binding to the DNA-binding-protein recognition site

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and a second fusion gene capable of expressing a second fusion protein, the second fusion protein comprising a polypeptide fragment of Smad3 covalently bonded to a gene activating moiety.

In preferred embodiments of the first eight aspects of the invention, a decrease in reporter gene expression indicates a compound that is capable of inhibiting TGF-β superfamily signalling, and an increase in reporter gene expression indicates a compound that is capable of enhancing TGF-β superfamily signalling. In other embodiments of these aspects of the invention, reporter gene expression may be assayed by a color reaction or assayed by cell viability. In still another embodiment of the first eight aspects of the invention, the cell may be a yeast cell.

In the ninth, tenth, eleventh, and twelfth aspects, the invention provides a method for detecting a compound capable of modulating TGF-β superfamily signalling. The method comprises the steps of providing a first polypeptide comprising a polypeptide fragment of FAST-1, providing a second polypeptide, the second polypeptide comprising a polypeptide fragment of either Smad2 or Smad3 (or alternatively, providing a first polypeptide comprising a polypeptide fragment of Smad2 or Smad3, and providing a second polypeptide comprising a polypeptide fragment of FAST-1), exposing the first polypeptide to the second polypeptide and to the compound, and measuring the level of interaction between the first polypeptide and the second polypeptide, wherein an alteration in the level of interaction indicates that the compound is capable of modulating TGF-β superfamily signalling.

In one preferred embodiment of the ninth, tenth, eleventh, and twelfth aspects of the invention, at least one of the first polypeptide or the second

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polypeptide is immobilized on a solid-phase substance. In another preferred embodiment, a decrease in the level of interaction indicates that the compound is capable of inhibiting TGF-β superfamily signalling, and an increase in the level of interaction indicates that the compound is capable of enhancing TGF-β

5   superfamily signalling.   In other embodiments of the ninth, tenth, eleventh, and twelfth aspects, the first polypeptide is produced by a cell that contains a first fusion gene capable of expressing the first polypeptide, and the second polypeptide is produced by a cell that contains a second gene capable of expressing the second polypeptide.

10   In various preferred embodiments of all of the above aspects of the invention, the polypeptide fragment of FAST-1 consists of, at maximum, *Xenopus* FAST-1 amino acids 380 to 506, human FAST-1 amino acids 234 to 365, and mouse FAST-1 amino acids 309 to 398. In other preferred embodiments of all of the aspects of the invention, the polypeptide fragment of Smad2 consists of, at

15   maximum, Smad2 amino acids 248 to 467 or 274 to 467, and the polypeptide fragment of Smad3 consists of, at maximum, Smad3 amino acids 230 to 446, amino acids 253 to 446, amino acids 230 to 424, or amino acids 253 to 424.

In the thirteenth aspect, the invention features a polypeptide comprising a polypeptide fragment of FAST-1. In a preferred embodiment of this aspect of

20   the invention, the polypeptide fragment of FAST-1 includes, at maximum, *Xenopus* FAST-1 amino acids 380 to 506, or fragments thereof, human FAST-1 amino acids 234 to 365, or fragments thereof, or mouse FAST-1 amino acids 309 to 398, or fragments thereof.

In the fourteenth, fifteenth, sixteenth, and seventeenth aspects, the

25   invention features a method for detecting a compound capable of modulating TGF-

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β superfamily signalling, comprising providing a reporter gene operably linked to a DNA-binding-protein recognition site and additionally providing either:

a) a first fusion protein comprising a polypeptide fragment of FAST-1 covalently bonded to a binding moiety capable of specifically binding to the DNA-binding-protein recognition site and a second fusion protein comprising a polypeptide fragment of Smad2 covalently bonded to a gene activating moiety,

b) a first fusion protein comprising a polypeptide fragment of Smad2 covalently bonded to a binding moiety capable of specifically binding to the DNA-binding-protein recognition site and a second fusion protein comprising a polypeptide fragment of FAST-1 covalently bonded to a gene activating moiety,

c) a first fusion protein comprising a polypeptide fragment of FAST-1 covalently bonded to a binding moiety capable of specifically binding to the DNA-binding-protein recognition site and a second fusion protein comprising a polypeptide fragment of Smad3 covalently bonded to a gene activating moiety, or

d) a first fusion protein comprising a polypeptide fragment of Smad3 covalently bonded to a binding moiety capable of specifically binding to the DNA-binding-protein recognition site and a second fusion protein comprising a polypeptide fragment of FAST-1 covalently bonded to a gene activating moiety;

exposing the first fusion protein to the second fusion protein, to the reporter gene, and to the compound; and measuring the reporter gene expression, a change in the reporter gene expression indicating a compound that is capable of modulating TGF-β superfamily signalling.

In various preferred embodiments of the fifteenth, sixteenth, seventeenth, and eighteenth aspects, a change in reporter gene expression that is a decrease indicates a compound that is capable of inhibiting TGF-β superfamily

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signalling, and a change in the reporter gene expression that is an increase in the reporter gene expression indicates a compound that is capable of enhancing TGF-β superfamily signalling. In other embodiments, the polypeptide of FAST-1 includes *Xenopus* FAST-1 amino acids 380 to 506, or fragments thereof, human FAST-1 amino acids 234 to 365, or fragments thereof, or mouse FAST-1 amino acids 309 to 398, or fragments thereof; the polypeptide fragment of Smad2 includes Smad2 amino acids 248 to 467, or fragments thereof; and the polypeptide fragment of Smad3 includes Smad3 amino acids 230 to 424, or fragments thereof. In yet another embodiment, providing the first fusion protein comprises providing a first fusion gene capable of expressing the first fusion protein and providing the second fusion protein comprises providing a second fusion gene capable of expressing the second fusion protein.

In the nineteenth aspect, the invention provides a method for diagnosing a mammal having or likely to develop a disorder involving abnormal TGF-β superfamily signalling. The method includes determining whether the mammal has a mutation in a gene encoding FAST-1. In a preferred embodiment of this aspect, the mutation is in the Smad Interaction Domain (SID).

In the twentieth aspect, the invention provides methods for diagnosing a mammal having or likely to develop a disorder involving abnormal TGF-β superfamily signalling comprising determining whether the mammal has an altered level of expression of FAST-1.

In preferred embodiments of the nineteenth and twentieth aspects of the invention, the disorder is a developmental disorder, and the mammal is a human, and may be a fetus.

In the twentieth aspect, the invention features a substantially pure

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mammalian FAST-1 protein or polypeptide fragment thereof for use in modulating TGF-β superfamily signalling.

In preferred embodiments of the twentieth aspect, the protein or polypeptide fragment may be from a human or a rodent. In other preferred embodiments, the polypeptide fragment comprises the Smad Interaction Domain (SID). In still another preferred embodiment, the polypeptide fragment binds to Smad2 or Smad3.

In a twenty-first aspect, the invention features a substantially pure polypeptide fragment comprising the Smad Interaction Domain (SID) of FAST-1 from *Xenopus*, for use in modulating TGF-β superfamily signalling.

In related, twenty-second, twenty-third, and twenty-fourth aspects, the invention features substantially pure polypeptides or fragments thereof having about 50% or greater amino acid sequence identity, about 75% or greater amino acid sequence identity, and about 90% or greater amino acid sequence identity to the comparable amino acid sequence of the mammalian FAST-1 protein or polypeptide fragment thereof. Preferably, the identity is determined by comparison with the FAST-1 SID (i.e., FAST-1 amino acids 380 to 509 of *Xenopus* FAST-1, amino acids 234 to 365 of human FAST-1, or amino acids 309 to 398 of mouse FAST-1). In another preferred embodiment, the polypeptide fragment binds to Smad2 or Smad3.

In a twenty-fifth aspect, the invention features a substantially pure nucleic acid encoding a mammalian FAST-1 protein or polypeptide fragment thereof.

In a twenty-sixth aspect, the invention features a vector containing a nucleic acid of the twenty-fifth aspect, capable of directing expression of the

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protein or polypeptide fragment thereof.

In a twenty-seventh aspect, the invention features a substantially pure nucleic acid encoding a FAST-1 Smad Interaction Domain (SID).

In a twenty-eighth aspect, the invention features a cell containing the vector of the twenty-sixth and twenty-seventh aspects above.

In a twenty-ninth aspect, the invention features a method of modulating TGF-β superfamily signalling in a cell, comprising providing a cell intracellularly with a substantially pure FAST-1 protein, or polypeptide fragment thereof, wherein the FAST-1 protein or polypeptide fragment is sufficient to modulate TGF-β superfamily signalling in a cell.

In a thirtieth aspect, the invention features a method of modulating TGF-β superfamily signalling in a cell, comprising introducing, into a cell, a vector comprising a nucleic acid encoding FAST-1 protein, or polypeptide fragment thereof, wherein the vector is capable of directing expression of the protein or polypeptide fragment in a cell containing the vector, and wherein expression of the FAST-1 protein or polypeptide fragment is sufficient to modulate TGF-β superfamily signalling in a cell.

In preferred embodiments of the twenty-ninth and thirtieth aspects, the signalling may be decreased or increased.

"Reporter gene" means any gene that encodes a product whose expression is detectable. Such genes include, without limitation, lacZ, amino acid biosynthetic genes, for example, the yeast LEU2, HIS3, LYS2, TRP1, or URA3 genes, nucleic acid biosynthetic genes, the mammalian chloramphenicol transacetylase (CAT) gene or GUS gene, or any surface antigen for which specific antibodies are available. Reporter genes may encode any enzyme that provides a

phenotypic marker, for example, a protein that is necessary for cell growth or a toxic protein leading to cell death, or gene encoding a protein detectable by color assay or whose expression leads to an absence of color. Other preferred reporter genes are those encoding fluorescent markers, such as the green fluorescent protein (GFP)-encoding gene, or reporter genes encoding enzymes whose activity may be detected by chemiluminescence, such as luciferase. Reporter genes may facilitate either a selection or a screen for reporter gene expression, and quantitative differences in reporter gene expression may be measured as an indication of interaction affinities.

"Covalently bonded" means that two domains are joined by covalent bonds, directly or indirectly. That is, the "covalently bonded" proteins or protein moieties may be immediately contiguous or may be separated by stretches of one or more amino acids within the same fusion protein.

"Protein" or "polypeptide" or "polypeptide fragment" means any chain of more than two amino acids, regardless of post-translational modification (e.g., glycosylation or phosphorylation), constituting all or part of a naturally-occurring polypeptide or peptide, or constituting a non-naturally occurring polypeptide or peptide.

"Smad2 protein or polypeptide fragment thereof" means a Smad2 protein (or polypeptide fragment or domain thereof) found in *Xenopus* or mammalian (e.g. mouse or human) cells. A preferred domain of Smad2 is the Mad Homology 2 (MH2) domain (i.e., amino acids 274 to 467 of human or *Xenopus* Smad2). Also preferred are polypeptide fragments comprising the MH2 domain, that consist of, at maximum, amino acids 274 to 467 or amino acids 248 to 467 of human or *Xenopus* Smad2, or the corresponding amino acids that comprise Smad2

MH2 domains from other species. These polypeptide fragments are capable of interacting with the FAST-1 Smad Interaction Domain (SID).

"Smad3 protein or polypeptide fragment thereof" means a Smad3 protein (or polypeptide fragment or domain thereof) found in *Xenopus* or

5 mammalian (e.g. mouse or human) cells. A preferred domain of Smad3 is the Mad Homology 2 (MH2) domain (i.e., amino acids 253 to 446 of human Smad3). Also preferred are polypeptide fragments comprising the MH2 domain, that consist of, at maximum, human Smad3 amino acids 230 to 446, and subfragments thereof, consisting of, at maximum, amino acids 253 to 446, amino acids 253 to 424, or

10 amino acids 230 to 424, or the corresponding amino acids that comprise Smad3 MH2 domains from other species. These polypeptide fragments are capable of interacting with the FAST-1 SID domain.

"Mammalian FAST-1 protein or polypeptide fragment thereof" means an amino acid sequence derived from a mammalian cell which displays at least

15 30%, preferably, 40%, more preferably 50%, still more preferably 60%, 70%, or even 80% means amino acid sequence identity to a FAST-1 Smad Interaction Domain (SID), i.e., amino acids 380 to 506 of the *Xenopus* FAST-1 protein, amino acids 234 to 365 of the human FAST-1 protein, or amino acids 309 to 398 of the mouse FAST-1 protein. The length of comparison, generally will be at least 16

20 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and most preferably at least 30 amino acids. Preferably, a mammalian FAST-1 protein, or polypeptide fragment thereof, is able to bind Smad2. The FAST-1 SID is a preferred polypeptide fragment of FAST-1.

"Operably linked" means that a gene and one or more regulatory

25 sequences are connected in such a way as to permit gene expression when the

appropriate molecules (e.g., transcriptional activator proteins) are bound to the regulatory sequences.

"Binding moiety" means a stretch of amino acids which is capable of directing specific polypeptide binding to a particular DNA sequence (i.e., a

5   "protein binding site").

"Modulatory compound" or "modulating compound", as used herein, means any compound capable of either increasing or decreasing the amount of signalling initiated by a TGF-β superfamily member.

"Substantially pure protein" or substantially pure polypeptide" means a

10   protein or polypeptide that has been separated from the components that naturally accompany it. Typically, the polypeptide is substantially pure when it is at least 60%, by weight, free from the proteins and naturally-occurring organic molecules with which it is naturally associated. Preferably, the polypeptide is a *xenopus* or mammalian, e.g. human or mouse, FAST-1 polypeptide, or polypeptide fragment

15   thereof, that is at least 75%, more preferably at least 90%, and most preferably at least 99%, by weight, pure. A substantially pure mammalian, e.g. human or mouse, FAST-1 polypeptide, or polypeptide fragment may be obtained, for example, by extraction from a natural source (e.g. a fibroblast, neuronal cell, or lymphocyte) by expression of a recombinant nucleic acid encoding a FAST-1

20   polypeptide, or by chemically synthesizing the protein. Purity can be measured by any appropriate method, e.g., by column chromatography, polyacrylamide gel electrophoresis, or HPLC analysis.

A polypeptide is substantially free of naturally associated components when it is separated from those contaminants which accompany it in its natural

25   state. Thus, a polypeptide which is chemically synthesized or produced in a

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cellular system different from the cell from which it naturally originates will be substantially free from its naturally associated components. Accordingly, substantially pure polypeptides include those which naturally occur in eukaryotic organisms but are synthesized in *E. coli* or other prokaryotes.

5   "Substantially pure nucleic acid" means nucleic acid that is free of the genes which, in the naturally-occurring genome of the organism from which the nucleic acid of the invention is derived, flank the gene. The term therefore includes, for example, a recombinant nucleic acid that is incorporated into a vector; into an autonomously replicating plasmid or virus; or into the genomic

10   nucleic acid of a prokaryote or eukaryote; or that exists as a separate molecule (e.g., a cDNA or a genomic or cDNA fragment produced by PCR or restriction endonuclease digestion) independent of other sequences. It also includes a recombinant nucleic acid that is part of a hybrid gene encoding additional polypeptide sequence.

15   By "Substantially identical" means a polypeptide or nucleic acid exhibiting at least 75%, preferably 85%, more preferably 90%, and most preferably 95% identity to a reference amino acid or nucleic acid sequence. For polypeptides, the length of comparison will generally be at least 16 amino acids, preferably at least 20 amino acids, more preferably at least 25 amino acids, and

20   most preferably 35 amino acids. For nucleic acids, the length of comparison sequences will generally be at least 50 nucleotides, preferably at least 60 nucleotides, more preferably at least 75 nucleotides, and most preferably 110 nucleotides.

        Sequence identity is typically measured using sequence analysis

25   software with the default parameters specified therein (e.g., Sequence Analysis

Software Package of the Genetics Computer Group, University of Wisconsin

Biotechnology Center, 1710 University Avenue, Madison, WI 53705). This

software program matches similar sequences by assigning degrees of homology to

various substitutions, deletions, and other modifications. Conservative

5   substitutions typically include substitutions within the following groups: glycine,

alanine, valine, isoleucine, leucine; aspartic acid, glutamic acid, asparagine,

glutamine; serine, threonine; lysine, arginine; and phenylalanine, tyrosine.

Default settings of sequence analysis software programs employ

parameters that are considered, by those of skill in the art, to yield biologically

10   significant results; i.e., an alignment of two polypeptides that shows one or more

amino acid stretches having a high percentage of sequence identity represents two

polypeptides that share a functional relationship. For example, FAST-1

polypeptides are identified by virtue of their possessing an amino acid sequence

that displays at least 30% identity to a FAST-1 SID.

15   Brief Description of the Drawings

Fig. 1 shows a schematic diagram indicating the interactions between

FAST-1, Smad2, and DPC4 (Smad4) in ARF formation. WH and SID indicate the

winged helix region and the Smad Interaction Domain of FAST-1, respectively.

Fig. 2 shows a supershifted electrophoretic mobility-shift assay (EMSA)

20   demonstrating the incorporation of Smad4 into the ARF complex.

Fig. 3 shows a supershifted EMSA demonstrating the presence of

Smad2 and Smad4 within the same ARF complex.

Fig. 4 shows a supershifted EMSA demonstrating that the Smad2/MH2

domain alone can be incorporated into the ARF complex.

Fig. 5A shows a Western blot of whole lysates and anti-GST co-immunoprecipitates from *Xenopus* embryos co-microinjected with RNA encoding GST-FAST-1 and Myc-Smad1 or Myc-Smad2, plus or minus RNA encoding activin, demonstrating that Smad2 but not Smad1 co-precipitates with FAST-1 in an activin-stimulated manner.

Fig. 5B shows a Western blot of whole lysates and anti-GST co-immunoprecipitates from *Xenopus* embryos co-microinjected with RNA encoding GST-FAST-1 and Myc-Smad4 (Myc-DPC4), plus or minus RNA encoding activin, demonstrating that Smad4 co-precipitates with FAST-1 in an activin-dependent manner.

Fig. 6A shows a summary of experiments that tested the ability of Myc-tagged FAST-1 deletion mutants to become incorporated into the ARF/ARE complexes or to associate with Smad2 in an activin-dependent and -independent manner.

Fig. 6B is a diagram of Myc-tagged FAST-1 showing the Smad Interaction Domain (SID) and the winged helix domain (amino acids 108-219).

Fig. 7 shows an EMSA demonstrating the inhibition of ARF/ARE complex formation by overexpression of the FAST-1 SID.

Fig. 8A shows an agarose gel containing RT-PCR amplification products that were generated using primers specific for the pan-mesodermal marker brachyury (Xbra), and the ubiquitously expressed marker EF1α, demonstrating that the FAST-1 SID inhibits activin-induced brachyury expression.

Fig. 8B (Panels A-F) shows a series of photographs of animal caps from control and experimentally-manipulated *Xenopus* embryos, demonstrating that the

FAST-1 SID blocks activin induction of mesodermal cell movements in early embryos.

Fig. 9 shows a Western blot analysis, with anti-Myc antibody, of whole lysates and FAST-1 immunoprecipitates from lysates of control embryos and embryos expressing GST-tagged FAST-1 plus Myc-tagged Smad1 MH2 domain or Myc-tagged Smad2 MH2 domain, demonstrating that the Smad2 MH2 domain co-immunoprecipitates FAST-1 in an activin-stimulated manner.

Fig. 10 shows an amino acid sequence alignment of human, mouse, and *Xenopus* FAST-1.

Detailed Description of the Invention

It is now demonstrated that the interactions of a FAST-1 polypeptide fragment with Smad2 and Smad3 polypeptide fragments *in vivo* as well as *in vitro* are clearly involved in TGF-β superfamily signalling pathways in eukaryotic cells.

In *Xenopus laevis* embryos, Smad2 is a component of the activin responsive factor (ARF) complex that binds to the ARE promoter element of the Mix.2 gene. The major DNA binding component of the ARF is a novel winged helix transcription factor that we have named FAST-1. In the present invention, we show that Smad4 is present in ARF, and that FAST-1, Smad4, and Smad2 co-immunoprecipitate in an activin-regulated fashion. We have mapped the site of interaction between FAST-1 and Smad2/Smad4 to a novel C-terminal domain of FAST-1; overexpression of this domain specifically inhibits activin signalling.

In a yeast 2-hybrid assay, the FAST-1 C-terminus was found to directly interact with Smad2, but not Smad4. Furthermore, we can detect binding of the

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FAST-1 C-terminus to the MH2 domain of Smad2 *in vitro*. The results of these findings have allowed us to propose the model for ARF formation shown in Fig. 1.

The interaction of FAST-1 and Smad2 domains provided in the present invention allows the identification of compounds capable of modulating the effects of TGF-β superfamily signalling and the identification of patients who either have or are likely to develop disorders involving abnormal TGF-β superfamily-mediated signal transduction.

I.   Uses for the Invention

The methods and compounds provided in the invention allow modulation and simulation of the signalling pathways of TGF-β superfamily members. These methods and compounds may provide a means to detect treatments and to possibly treat or cure individuals with a variety of diseases, including, without limitation, developmental disorders, immunological disorders, and cancer. The invention also describes methods by which individuals may be identified who either have or are likely to develop disorders involving abnormal TGF-β superfamily signalling.

II.   FAST-1, Smad2, and Smad3 Fragments

We have found that polypeptide fragments comprising various portions of the FAST-1, Smad2 and Smad3 proteins have been useful in identifying the domains important for the interaction of FAST-1 (SEQ ID NO: 11, 14, and 17) with either Smad2 (SEQ ID NO: 2 and 5) or Smad3 (SEQ ID NO: 8). Methods for generating such fragments are well known in the art (see, for example, Ausubel et al., Current Protocols in Molecular Biology, John Wiley & Sons, New York,

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NY, 1994) and are further described herein. For example, a Smad2 polypeptide fragment may be generated by amplifying the desired fragment by the polymerase chain reaction (PCR) using oligonucleotide primers designed according to the published Smad2 nucleic acid sequence (SEQ ID NO: 1 and 4). Preferably the oligonucleotide primers comprise unique restriction enzyme sites which facilitate insertion of the DNA fragment into the cloning site of a eukaryotic expression vector. Furthermore, the Smad2 fragment may be tagged with an epitope (e.g., hemagglutinin (HA) or GST) by cloning the fragment into a epitope fusion expression vector. The Smad2 fragment-bearing vector is then introduced into a prokaryotic or eukaryotic cell by artifice, using the various techniques known in the art and described herein, which result in the production of the Smad2 polypeptide fragment. Similar techniques using FAST-1 (SEQ ID NO: 10, 13, and 16) and Smad3 (SEQ ID NO: 7) nucleic acid sequences are employed to generate FAST-1 and Smad3 polypeptide fragments.

In one approach, FAST-1 and Smad2, or Smad3 polypeptide fragments may be used to evaluate the portions of these proteins involved in regulation of TGF-β signalling during *Xenopus laevis* embryogenesis. In particular, polypeptide fragments comprising the domains of the FAST-1, Smad2, and Smad3 proteins responsible for the interaction of FAST-1 with either Smad2 or Smad3 may be used to induce TGF-β superfamily signalling, or to prevent TFG-β superfamily signalling.

III. Screens for Compounds Which Modulate TGF-β Superfamily Signalling

FAST-1 and Smad2 or FAST-1 and Smad3 may be used to facilitate the identification of compounds that increase or decrease TGF-β superfamily-

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mediated signal transduction. In one approach, compounds that modulate the signals generated by the TGF-β superfamily are detected by screening for compounds that alter the physical interaction between the FAST-1 SID domain (SEQ ID NO: 12, 15, and 18) and the Smad2 (SEQ ID NO: 3 and 6) or Smad3

5   (SEQ ID NO: 9) MH2 domain. These compounds are detected by adapting yeast two-hybrid expression systems known in the art for use as described herein. These systems which allow detection of protein interactions via a transcriptional activation assay, are generally described by Gyuris et al. (Cell 75:791-803, 1993) and Fields et al. (Nature 340:245-246, 1989), and are commercially available from

10   Clontech (Palo Alto, CA).

       In this approach, a region of FAST-1, which we have discovered interacts with Smad2, is fused to the GAL4-DNA-binding domain by subcloning a DNA fragment encoding this, the FAST-1 Smad Interaction Domain (SID), into the expression vector, pGBT9, provided in the MATCHMAKER Two-Hybrid

15   System kit commercially available from Clontech (catalog number K1605-1). A fusion of the GAL4 activation domain with the MH2 domain of Smad2 or Smad3 (which interacts with the FAST-1 SID) is generated by subcloning the Smad2 or Smad3 MH2 domain-encoding DNA fragment into the expression vector, PGAD424, also provided in the Clontech kit. Analogous expression vectors may

20   also be used. Yeast transformations and colony lift filter assays are carried out according to the methods of MATCHMAKER Two-Hybrid System and various methods known in the art. Prior to the colony filter assay, transformed yeast may be treated with candidate compounds being screened for the ability to modulate TGF-β signalling. The interaction results obtained using the candidate compound

25   in combination with the yeast system may then be compared to those results

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observed with the yeast system not treated with the candidate compound, all other factors (e.g., cell type and culture conditions) being equal. A compound capable of modulating TGF-β superfamily-mediated signalling is able to alter the interaction between the Smad2 or Smad3 MH2 domain and the FAST-1 SID.

5   In another embodiment of this approach, a compound capable of decreasing TGF-β superfamily signalling by disrupting the binding of the Smad2 (or Smad3) MH2 to the FAST-1 SID may be isolated using the modified yeast-two hybrid system described above, in which the reporter gene encodes a protein, such as ricin, that is toxic to yeast. Yeast cells containing such a ricin reporter gene die

10   unless the binding of Smad2 MH2 to FAST-1 SID is disrupted. Yeast cells treated with a compound that disrupts the Smad2/FAST-1 interaction form viable colonies, and from this result it may be inferred that the compound is capable of decreasing, and possibly inhibiting, signals initiated by members of the TGF-β superfamily.

15   In another approach, compounds capable of inhibiting signalling by TGF-β and other members of the TGF-β superfamily may be identified *in vitro* using assays that detect disruption of the *in vitro* binding of FAST-1 SID to the Smad2 (or Smad3) MH2 domain. For example, in order to detect FAST-1/Smad interactions, the FAST-1 SID domain is fused to glutathione S-transferase (GST)

20   by subcloning the FAST-1 SID-encoding DNA fragment into a bacterial expression vector that encodes a GST tag. Such vectors are well known in the art and are commercially available (e.g., the pGEX fusion vectors commercially available from Pharmacia). GST-tagged FAST-1 SID fusion protein is produced by transforming the GST-FAST-1 SID-encoding vector into *E. coli* bacteria.

25   Fusion proteins are then purified by allowing the proteins from lysed bacteria to

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bind to glutathione sepharose-coated beads. The GST-tagged FAST-1 SID-bearing beads are then used to specifically bind Myc-tagged Smad2 (or Smad3) MH2 domains polypeptides produced in *Xenopus* embryos. Detection of FAST-1/Smad2 (or Smad3) interactions are assessed by resolving the glutathione-immobilized proteins by Laemmli gel electrophoresis and subjecting the resolved proteins to Western blot analysis using anti-Myc antibodies.

In order to detect compounds that inhibit TGF-β superfamily signalling by disrupting FAST-1/Smad2 (or Smad3) interactions, *Xenopus* embryo lysates containing Myc-tagged Smad2 (or Smad3) MH2 domain polypeptides are incubated with a candidate TGF-β signalling modulatory compound prior to the incubation with glutathione Sepharose-coated beads carrying the GST-tagged FAST-1 SID. Glutathione-immobilized proteins from treated *vs.* untreated *Xenopus* embryo lysates are then subjected to Western blotting with anti-Myc antibodies. A difference in the amount of anti-Myc reactivity of the glutathione-immobilized proteins from treated samples *vs.* untreated samples indicates that the test compound modulates TGF-β superfamily-mediated signal transduction.

GST-tagged FAST-1 SID fusion proteins may be immobilized on a solid-state substance for rapid high-throughput identification of compounds that modulate TGF-β superfamily signalling. Preferably, the solid-state substance is the bottom of a well on a 96-well (or similar) plate. Each well may then be provided with a known amount of the MH2 domain of either Smad2 or Smad3 that is tagged with a readily detectable epitope (e.g., an short polypeptide fragment, e.g., HA or Myc, that is specifically recognized by an antibody). Preferably, a Smad2 or Smad3 MH2 domain tagged with the alkaline phosphatase (AP) enzyme is added to each GST-tagged FAST-1 SID-bearing well. Candidate compounds to

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be screened for an ability to modulate TGF-β superfamily signalling are then added individually or in combination to each well on the plate. After allowing the interaction of the components in each well, the plate is washed, and the substrate for AP is added to each well. A compound that modulates TGF-β superfamily

5   signalling may affect the binding affinities of the FAST-1 SID and the Smad2 or Smad3 MH2 such that the amount of bound Smad MH2, and hence, bound AP enzymatic activity, is altered. Preferable AP substrates are colorimetric substrates, such as the nitro blue tetrazolium (NBT) and 5-bromo-1-chloro-3-indolyl-phosphate (BCIP) reagents that are commercially available (e.g., from Promega).

10   After allowing formation of the blue/black precipitate to occur in a control well that has not been treated with a candidate compound, the plate is quantitated for color intensity on a 96-well plate reader. A compound that affects the color intensity of AP substrates when added to a well, as compared to a well not treated with a compound, indicates a compound that has the ability to modulate

15   TGF-β superfamily mediated signal transduction.

Molecules that are found to effectively modulate TGF-β superfamily signalling, using the methods described above, may be further tested using *in vivo* animal models. Compounds that function effectively in an *in vivo* setting may be used as therapeutics to either inhibit or enhance TGF-β superfamily member-

20   mediated signalling, as appropriate.

IV. Administration of Modulators of TGF-β Superfamily Signal Transduction

A TGF-β superfamily signalling modulator may be administered within a pharmaceutically-acceptable diluent, carrier, or excipient, in unit dosage form. Conventional pharmaceutical practice may be employed to provide suitable

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formulations or compositions to administer a TGF-β superfamily signalling modulator(s) to patients suffering from a disease (e.g., a developmental disease) that is caused by an abnormal amount of TGF-β superfamily member-mediated signal transduction. Administration may begin before the patient is symptomatic.

5 Any appropriate route of administration may be employed, for example, administration may be parenteral, intravenous, intra-arterial, subcutaneous, intramuscular, intracranial, intraorbital, ophthalmic, intraventricular, intracapsular, intraspinal, intracisternal, intraperitoneal, intranasal, aerosol, by suppositories, or oral administration. Therapeutic formulations may be in the form of liquid

10 solutions or suspensions; for oral administration, formulations may be in the form of tablets or capsules; and for intranasal formulations, in the form of powders, nasal drops, or aerosols.

Methods well known in the art for making formulations are found, for example, in "Remington's Pharmaceutical Sciences." Formulations for parenteral

15 administration may, for example, contain excipients, sterile water, or saline, polyalkylene glycols such as polyethylene glycol, oils of vegetable origin, or hydrogenated napthalenes. Biocompatible, biodegradable lactide polymer, lactide/glycolide copolymer, or polyoxyethylene-polyoxypropylene copolymers may be used to control the release of the compounds. Other potentially useful

20 parenteral delivery systems for TGF-β superfamily signalling modulatory compounds include ethylene-vinyl acetate copolymer particles, osmotic pumps, implantable infusion systems, and liposomes. Formulations for inhalation may contain excipients, for example, lactose, or may be aqueous solutions containing, for example, polyoxyethylene-9-lauryl ether, glycocholate and deoxycholate, or

25 may be oily solutions for administration in the form of nasal drops, or as a gel.

Dosage is determined by standard techniques and is dependent, for example, upon the weight of the patient and the type or extent of disorder being treated.

V. Diagnostics for Disorders involving Abnormal TGF-β Superfamily Signalling

To determine whether an individual either has or is likely to develop a disorder (e.g., a developmental disorder) involving abnormal TGF-β superfamily signalling, that individual may be screened for mutations in the domains (e.g., the SID of FAST-1 or the MH2 domains of Smad2 and Smad3) of the genes encoding FAST-1, Smad2, and Smad3 that mediate the binding interactions of FAST-1 with Smad2 or Smad3. Screening for mutations may be carried out using any standard technique including, without limitation, methods involving sequencing, or mismatch binding or cleaving assays. For example, a nucleic acid sample may be derived from cells of an individual to be tested for a mutation (for example, by PCR amplification), and the FAST-1, Smad2, and Smad3 genes may be subjected to rapid sequence analysis by automated sequencing techniques using primers generated from FAST-1, Smad2, and Smad3 sequences described in the art and herein.

Alternatively, an individual who either has or is likely to develop a disorder involving abnormal TGF-β superfamily signalling may be screened for altered expression of FAST-1, Smad2 or Smad3. Such assays may be carried out, for example, using any standard nucleic acid-based assay (e.g., Northern blot analysis) or immunological assay (e.g., enzyme-linked immunosorbent assay (ELISA)), preferably in a high through-put assay format. For example, cells may be obtained from an individual to be tested, and analyzed by ELISA for the

expression of FAST-1, Smad2, or Smad3 proteins, using as probes, fluorophore-tagged antibodies directed against these proteins. Individuals that have altered protein levels relative to the general population, are readily identified using such ELISA-based assays.

5   VI. FAST-1 Related Genes

Standard techniques, such as the polymerase chain reaction (PCR) and DNA hybridization, may be used to clone additional FAST-1 homologues in other species. In order to detect such homologues, genomic DNA of various organisms (e.g., humans or mice) may be analyzed by Southern blotting using nucleic acid

10   probes generated from the nucleic acid sequences encoding *Xenopus* FAST-1. Hybridization at low stringency should reveal bands that correspond to DNA encoding FAST-1 and/or related family members. *Xenopus* FAST-1 nucleic acid probes may be based upon the codon preference of the organism, whose DNA is under analysis, or they may be degenerate probes based upon all possible codon

15   combinations, or they may be a combination of codon preference and codon degeneracy. Such probes may also be used to screen either genomic or cDNA libraries for sequences that hybridize to the probe. FAST-1 nucleic acid probes also may be used as primers to clone additional FAST-1 related genes by RT-PCR, using methods known in the art.

20   Another method for identifying mammalian homologues of the FAST-1 is by searching publically available databases for sequences that share sequence identity with the *Xenopus* FAST-1 nucleic acid or amino acid sequence (Genbank accession number U70980), or with sequence fragments thereof. A particularly preferred FAST-1 sequence fragment is the sequence corresponding to the Smad

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Interaction Domain (SID) of FAST-1. Once identified, a candidate mammalian homologue of FAST-1 (or polypeptide fragment thereof) may be tested for FAST-1-like activity (e.g., ability to bind the Smad2 or Smad3 MH2 domain), using the assays described herein.

5   The following examples are meant to illustrate the invention. They are not meant to limit the invention in any way.

EXAMPLE I

Smad4 (DPC4) is a component of ARF.

Signalling by TGF-β superfamily members induces transcriptional

10   activation of target genes. Some of these transcriptional responses are necessary and sufficient for the specification or patterning of mesoderm. Several TGF-β superfamily-responsive genes show immediate-early responses, ie., they are induced even when translation is inhibited by cycloheximide. Two such genes, Mix.1 and Mix.2, are transcriptionally activated by signals initiated by TGF-β

15   superfamily members such as activin, Vg-1, TGF-β, and BMP4. In contrast, Mix.1 and Mix.2 are not transcriptionally activated by non-TGF-β mesoderm inducers or axial modifiers.

An activin-responsive factor (ARF) was identified using an electrophoretic mobility-shift assay (EMSA) for embryonic proteins that bind to

20   the Mix.2 promoter elements. The ARF, which is induced in embryonic blastomeres after 5-30 minutes of activin stimulation, binds specifically to a 50-bp Mix.2 promoter element. FAST-1 was identified as the major DNA-binding component of the ARF complex.

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Smad2, which associates in a ligand regulated manner with another member of the Smad family, Smad4 (DPC4), is a compound of the ARF complex. Therefore, we asked whether Smad4 is also a component of ARF.

Methods

5   HA-tagged Smad4 was provided by Akiko Hata and Joan Massague, and the untagged full length Smad4 construct was provided by P. Hoodless and J. Wrana. We have previously described the *Xenopus* activin encoding construct (Thomsen et al., Cell 63: 485-493). These constructs were *in vitro* transcribed according to standard techniques described in, for example, Krieg and Melton

10   (Meth. Enzymol. 155: 397-415, 1987).

      Xenopus laevis embryos at the 2-cell stage were microinjected in both blastomeres with 0.5-2 ng of RNA encoding HA-tagged or untagged Smad4 (DPC4) with or without RNA encoding activin, as indicated in Fig. 2. Embryos were maintained in 1X MMR containing 3% Ficoll during microinjection, after

15   which embryos were transferred to 0.1X MMR. Embryos were harvested for EMSA lysates at Stage 9 as previously described (Huang et al., EMBO J 14:5965-5973, 1995). Staging of embryos was done according to Nieuwkoop and Faber (Normal Table of *Xenopus laevis* (Daudin), Second edition ed. North Holland Publishing Company, Amsterdam, 1967).

20   EMSA was performed as previously described (Huang et al., *supra*) using as a probe the ^{32}P-labeled ARE from the Mix.2 promoter (Chen, et al., *Nature* 383:691-696, 1996). For supershift assays, EMSA assay mixtures were incubated with anti-HA antibody (commercially available from Gibco-BRL) for 1 hour on ice prior to SDS-PAGE and autoradiography.

Results

Fig. 2 shows a supershifted electrophoretic mobility-shift assay (EMSA) demonstrating the incorporation of Smad4 into the ARF complex. HA-tagged (lanes 2, 3, 7, and 8) or untagged (lanes 5 and 6) Smad4 was expressed in early

5   *Xenopus* embryos and incorporation of HA-tagged protein into ARF was tested by co-incubation of EMSA mixtures with anti-HA antibody (lanes 3, 6, and 8). "Activin" indicates samples in which activin was co-expressed. "ssARF" (super-shifted ARF) indicates increased mobility of the anti-HA antibody-bound ARF.

10   The EMSA experiment of Fig. 2 shows that Smad4 (DPC4) is a component of the ARF complex. A supershift of the ARF complex by anti-HA antibody is dependent upon the presence of HA-Smad4 within the embryo lysate (Fig. 8, lane 2). However, overexpression of Smad4 in the absence of stimulation by activin is not sufficient for ARF formation, since supershifts were detected only

15   in embryos co-injected with HA-Smad4 RNA plus activin RNA, but not in embryos injected with HA-Smad4 RNA alone. Hence, the binding of ligand (in this case, activin) to a TGF-β superfamily receptor appears to provide additional signals that are necessary for ARF formation.

EXAMPLE II

20   Smad4 (DPC4) and Smad2 co-associate in ARF complexes.

Incorporation of Smad4 (DPC4) into ARF might reflect the co-association of Smad2, Smad4, and FAST-1 within the same complex. Alternatively, there might be two types of ARF: a Smad2-containing ARF, which would predominate in the presence of overexpressed Smad2, and a Smad4-

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containing ARF, which would predominate in the presence of overexpressed Smad4. We next determined which of these two models was correct.

Methods

Xenopus Smad2 (provided by J. Graff and D. Melton) carrying six
5 consecutive Myc epitope tags at the Smad2 N-terminus was generated by cloning the Smad2 coding region into the fusion vector pCS2(+)MT, which encodes the Myc tags (Thomsen et al., Cell 63: 485-493, 1990; Turner and Weintraub, Gen. and Dev. 8: 1434-1447, 1994). Smad3 was similarly N-terminally fused to six Myc tags.

10 RNAs encoding Myc-tagged Smad2, HA-tagged Smad4, and activin were co-injected into two-cell *Xenopus laevis* embryos according to the method described in Example I. Stage 9 embryos were harvested and assayed by supershift-EMSA with anti-Myc or anti-HA antibodies as described in Example I.

Results

15 Fig. 3 shows a supershifted EMSA demonstrating the presence of Myc-tagged Smad2 and HA-tagged Smad4 within the same ARF complex. ARF complexes that were supershifted using only anti-HA antibody are designated "HA-ssARF", those using only anti-Myc antibody, "Myc-ssARF", and those that were supershifted using both antibodies are designated HA+Myc-ssARF.

20 The addition of both anti-HA and anti-Myc antibodies resulted in a more highly supershifted ARF, relative to supershifted ARFs produced by either antibody alone. This result strongly suggests that Smad2 and Smad4 co-exist within the same ARF complex, rather than within two discrete subsets of ARF

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complexes. We obtained identical results Myc- tagged Smad3 in place of Myc-tagged Smad2. Consistent with these results, we observed that the simultaneous addition of anti-HA and anti-Myc antibodies to lysates from embryos expressing HA-tagged Smad4 plus untagged Smad2, or Myc-tagged Smad2 plus untagged

5   Smad4, resulted in supershifted complexes analogous to those produced by using only one anti-epitope antibody.

EXAMPLE III

The Smad2 MH2 domain alone can be incorporated into the ARF complex

The Smad2 Mad Homology 2 (MH2) domain is necessary for Smad2-

10   dependent transcriptional activation. In order to determine whether the MH2 domain is also necessary for incorporation of Smad2 into the ARF, we used supershift-EMSA to ask whether the Smad2 MH2 domain alone could be incorporated into ARF complexes.

Methods

15   The Smad2 MH2 domain was tagged with the FLAG epitope by PCR-amplifying a DNA sequence encoding the Smad2 MH2 domain (Smad2 amino acids 248-467) and subcloning the PCR product into the pCS2+ vector (previously described by Thomsen et al., Cell 63: 485-493, 1990; and Turner and Weintraub, Gen. and Dev. 8: 1434-1447, 1994).

20   FLAG-tagged Smad2 MH2, HA-tagged Smad4, and activin were co-expressed in two-cell *Xenopus laevis* embryos according to the method described in Example I. Stage 9 embryos were harvested and assayed for incorporation into

the ARF by supershift-EMSA using anti-FLAG or anti-HA antibodies, as described in Example I.

Results

Fig. 4 shows a supershifted EMSA demonstrating that the Smad2 MH2 domain alone can be incorporated into the ARF. ARF complexes that have increased mobility due to the incorporation of Smad2-MH2 (rather than full-length Smad2) are indicated by "MH2-ARF", FLAG-Smad2-MH2-ARF complexes supershifted with anti-FLAG are indicated by "FLAG-ssMH2ARF", and Smad2-MH2 -ARF complexes supershifted by anti-HA (recognizing Smad4 in the complex) are indicated by "HA-ssMH2ARF".

The Smad2 MH2 domain was part of a complex that bound ARE, but that migrated more rapidly than did endogenous ARF (presumably due to the reduced molecular weight of complexes containing the Smad2 MH2 domain as opposed to full-length Smad2 (Fig. 4). Incorporation of the Smad2 MH2 domain into ARF was activin-dependent (Fig. 4, lanes 2 and 4). ARF complexes that contain the Smad2 MH2 domain are supershifted by anti-HA antibody, indicating that these complexes also contain HA-tagged Smad4 (Fig. 4, Lane 9).

EXAMPLE IV

Co-immunoprecipitation of FAST-1 with Smad2

In the experiments described in the previous examples, EMSA was used to detect the binding of ARF to its DNA target, ARE. To study FAST-1/Smad2 interactions in the absence of ARF/ARE complex formation, we asked whether

FAST-1 and Smad2 could be co-immunoprecipitated from *Xenopus* embryo lysates.

Methods

Myc-tagged Smad1 was generated by subcloning the sequences

5   encoding Smad1 from the FLAG-tagged Smad1 construct into the pCS2(+)MT vector, which is a modification of the pCS2+ vector originally described in Thomsen et al., *supra* and Turner and Weintraub, *supra*. FAST-1 was tagged by N-terminal fusion at amino acid 61 with GST.

Xenopus laevis embryos at the 2-cell stage were co-injected either with

10   RNA encoding GST-tagged FAST-1 plus RNA encoding Myc-tagged Smad1, or with RNA encoding GST-tagged FAST-1 plus RNA encoding Myc-tagged Smad2, both plus or minus co-injection of RNA encoding activin.

Embryos were harvested at Stage 9 in lysate buffer (as described in Example I), and cleared by centrifugation for 15 minutes at 32,000x g. Cleared

15   lysates were immunoprecipitated with anti-GST tag antibody for 1 hr at 4°C, and then incubated with protein A-sepharose for 30 min. The beads were then washed under the following conditions: 1X lysate buffer 0.1% NP40, 1X lysate buffer + 0.4M NaCl, 1X lysate buffer + 0.5% NP40, 1X lysate buffer + 0.2M NaCl, 0.25%NP40, 1X lysate buffer. Samples were fractionated by electrophoresis and

20   transferred to nitrocellulose. The nitrocellulose-immobilized immunoprecipitates were blotted with anti-Myc antibody and immunoreactive bands were detected by ECL as previously described (see LaBonne et al., Development 121: 1475-1486, 1995). In parallel, whole-embryo lysates were also subjected to electrophoresis, transferred to nitrocellulose, blotted with anti-Myc antibody, and subjected to ECL

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detection.

Results

As shown in Fig. 5A, Myc-tagged Smad2 co-immunoprecipitated with GST-tagged FAST-1, and Smad2/FAST-1 complex formation was enhanced by

5   activin stimulation (lanes 6 and 7). The anti-GST antibody did not immunoprecipitate Myc-tagged Smad2 from lysates of embryos not expressing GST-tagged FAST-1, indicating that Smad2 immunoprecipitation resulted from its specifically interacting with FAST-1.

In contrast to Smad2 and FAST-1, co-expression of tagged Smad1 and

10   FAST-1 did not lead to detectable co-immunoprecipitation of Smad-1 (Fig. 5A, lanes 3 and 4). Equivalent expression of Smad1 and Smad2 in embryos was confirmed by Western analysis of whole embryonic lysate (Fig. 5A, lower panel). Therefore, it appears that Smad1 and FAST-1 do not directly interact, or do not interact as strongly as do Smad2 and FAST-1.

15   Ligand-induced activation of TGF-β superfamily receptors is apparently not a prerequisite for Smad2/FAST-1 complex formation: Myc-tagged Smad2 co-immunoprecipitated with FAST-1 even in the absence of stimulation by activin, although Smad2/FAST-1 complex formation increased in lysates from embryos injected with activin mRNA. At high levels of FAST-1 expression, Smad2 co-

20   precipitation was nearly equivalent in lysates from activin-stimulated and unstimulated embryos (similar results were obtained using Myc-tagged Smad3 in place of Smad2, or by first immunoprecipitating Smad2 and then detecting co-immunoprecipitated FAST-1 by Western blot analysis).

We were surprised to observe the activin-independent co-precipitation

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of Myc-Smad2 and GST-FAST-1, since EMSA experiments described in previous examples showed that supershifting of ARF/ARE complexes by anti-Myc (i.e. Myc-Smad2) antibody was activin-dependent. This result suggests that in addition to the activin-dependent formation of FAST-1/Smad2-containing complexes that

5   are competent for DNA binding, there exist activin-dependent complexes that are either not competent for DNA binding, or not stable under our EMSA assay conditions.

EXAMPLE V

Co-precipitation of FAST-1 with Smad4 (DPC4)

10   In order to study the nature of FAST-1/Smad4 (DPC4) interacterations prior to ARE/ARF complex formation, we asked whether Smad4 and FAST-1 could be co-immunoprecipitated from activin-stimulated and unstimulated *Xenopus* embryo lysates.

Methods

15   Myc-tagged Smad4 was constructed by cloning the full-length Smad4 into the pCS2(+)MT vector, which is described in Example II.

Myc-tagged Smad4 was co-expressed with GST-tagged FAST-1 in *Xenopus* embryos in the presence or absence of activin stimulation. The microinjected embryos were lysed, GST-tagged FAST-1 was immunoprecipitated

20   with anti-GST antibody, and immunoprecipitates were subjected to Western blot analysis using anti-Myc antibody, as described in Example IV.

Results

Myc-tagged Smad4 (DPC4) was co-immunoprecipitated with GST-tagged FAST-1 from lysates of activin-stimulated embryos. However, such Smad4/FAST-1 complexes were not evident above background in lysates from unstimulated embryos (Fig. 5B). Identical results were obtained by

5　immunoprecipitating Myc-tagged Smad4 and performing immunoblots using an anti-GST (GST-FAST-1) antibody, or by substituting FLAG-tagged FAST-1 for GST-tagged FAST-1. Hence, the association of Smad4 with FAST-1 requires prior activin stimulation.

EXAMPLE VI

10　Deletion Analysis of FAST-1

FAST-1 contains a predicted winged helix DNA binding domain, but has no extensive homologies to other winged helix factors or other known proteins outside the DNA binding domain. To identify the regions of FAST-1 that are important for its incorporation into ARF, we expressed epitope-tagged deletion

15　mutants of FAST-1 in early embryos and tested them for incorporation into ARF by antibody supershift-EMSA.

Methods

FAST-1 was tagged by N-terminal fusion at amino acid 61 with 6 Myc tags, by cloning FAST-1 cDNA encoding amino acids 61 to 534 into the

20　pCS2(+)MT vector (see Example II) to generate the Myc-tagged 61-534 FAST construct. The Myc-tagged Δ1-366 FAST-1 construct was generated by cloning FAST-1 cDNA encoding amino acids 366 to 534 into the pCS2(+)MT vector.

Various deletion mutants of FAST-1 were constructed from the Myc-

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tagged FAST 61-534: Myc-tagged Δ516-534; Myc-tagged Δ506-534; Myc-tagged Δ473-534; Myc-tagged Δ281-366; Myc-tagged Δ366-380; Myc-tagged Δ366-407; Myc-tagged Δ207-453; Myc-tagged Δ366-473; Myc-tagged Δ366-534; Myc-tagged Δ380-407; and Myc-tagged Δ453-506. Messenger RNAs encoding the

5   Myc-tagged FAST-1 deletion mutants plus mRNA encoding activin were microinjected into two-cell *Xenopus laevis* embryos. Stage 9 embryos were harvested for EMSA as described in Example I, and the ability of anti-Myc antibodies to supershift ARF/ARE complexes was assessed.

Messenger RNAs encoding FAST-1 deletion mutants also were co-
10   microinjected either with RNA encoding Myc-tagged Smad2 plus or minus RNA encoding activin, or with RNA encoding HA-tagged Smad4 (DPC4) plus RNA encoding activin, and EMSA lysates were prepared from stage 9 embryos. ARF/ARE complexes that were supershifted by anti-Myc- or anti-HA-specific antibodies indicated FAST-1 deletion mutants that retained the ability to associate
15   with Smad2 or Smad4, respectively.

Results

In order to determine which regions of FAST-1 interact with Smad2 and Smad4 and which are necessary for incorporation into ARF and for ARF/ARE complex formation, mRNAs encoding epitope-tagged FAST-1 deletion mutants
20   were co-expressed with tagged Smad2 or Smad4 plus or minus activin. The summarized results of these experiments are shown in Fig. 6A, and FAST-1 polypeptide domains that are necessary for interactions with Smads and for ARF/ARE complex formation are shown in Fig. 6B (TAG=Myc tag; amino acids 108-219 delineate the winged helix domain).

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Deletions N-terminal to the forkhead domain (N-terminal to FAST-1 amino acid 107) do not appear to reduce incorporation of FAST-1 into ARF. Moreover, deletion of the N-terminal two-thirds of FAST-1 (up to amino acid 365), including the entire winged helix domain, does not reduce ligand-dependent

5   association of FAST-1 with Smad2 or Smad4 and, hence, does not reduce incorporation of FAST-1 into ARF.

However, as shown in Fig 6A, FAST-1 deletion mutants that lack the winged helix DNA-binding domain are not incorporated into ARF/ARE complexes. These data show that the winged helix domain is not necessary for

10   FAST-1/Smad interactions and for FAST-1 incorporation into the ARF, but is necessary for ARF/ARE complex formation.

Deletions from the N-terminal side of the C-terminal half of FAST-1, up to amino acid 380 (e.g., Δ 281-366), also allowed association of FAST-1 with Smad2 or Smad4 in an activin-dependent manner, as did a 29-amino acid C-

15   terminal deletion. However, deletion of an additional 33 C-terminal amino acids prevented association of FAST-1 with Smad2 or Smad4. Although deletions of the C-terminus beyond the C-terminal 29 amino acids prevents FAST-1 incorporation into ARF, such deletions do not affect the ARE-binding activity of FAST-1 itself.

20   The FAST-1 domain responsible for co-precipitation with Smad2 or Smad4 localizes to a 126 amino acid C-terminal domain (380-506); this domain also is necessary for incorporation of FAST-1 into ARF/ARE complexes. We call this region of FAST-1 the Smad Interaction Domain (SID). Additional FAST-1 deletions (Δ207-453, Δ506-518, and Δ473-518) allowed us to delimit the region

25   necessary for activin-dependent association of FAST-1 and Smad2 to amino acids

453-506. Experiments using a construct with a deletion (Δ366-473) from the N-terminal side of the SID showed that amino acids C-terminal to position 473 are sufficient for reduced, but still significant, activin-dependent association of FAST-1 with Smad2, but are insufficient for mediating interactions with Smad4,

5   or for ARF/ARE complex formation.

Comparison of the regions of FAST-1 necessary for ARF/ARE formation with those regions necessary for co-immunoprecipitation with Smad2 and with Smad4 revealed a subregion of the FAST-1 SID (amino acids 380-407) that was necessary for activin-dependent ARF/ARE complex formation, but not

10   necessary for the co-precipitation of Smad2 with FAST-1. However, this region was necessary for the co-immunoprecipitation of Smad4 with FAST-1. This finding, in combination with the observation that there were no deletion mutants of FAST-1 that co-immunoprecipitated with Smad4 but not with Smad2 in an activin-independent manner, suggests that Smad2 and FAST-1 initially interact in an

15   activin-independent manner (i.e., prior to engagement of the TGF-β receptor by ligand), and that activin-stimulated phosphorylation of the Smad2 C-terminus enhances the association between the Smad2 MH2 domain and the region of FAST-1 encompassing amino acids 453-506, as determined from experiments using mutants Δ366-407, Δ380-407, and Δ207-453. This activin-dependent step

20   allows Smad4 to interact with Smad2 and FAST-1.

Although FAST-1/Smad2 interaction occurs in the absence of Smad4 binding of the FAST-1/Smad2 complex to the ARE is not observed for FAST-1 mutants that are unable to bind Smad4. Hence, interaction among Smad4, Smad2, and a FAST-1 domain encompassing amino acids 380-407 is necessary for the

25   formation of stable ARF/ARE complexes.

To summarize, the FAST-1 SID appears to possess two subdomains that mediate the sequential association of FAST-1 with Smad2, which then associate with Smad4; ARF complexes must contain these three factors in order to stably bind the ARE.

5   EXAMPLE VII

Yeast-Two Hybrid Interaction of FAST-1 with Smads

The activin-stimulated co-precipitation of FAST-1 with Smad2 and Smad4 demonstrated that these polypeptides are able to form a complex in the absence of the ARE DNA target, but did not address whether additional

10   components of the activin signalling pathway are necessary for complex formation. In order to directly examine the physical interactions among Smad2, Smad4, and FAST-1, we tested these proteins for association in a yeast interaction trap system (Fields, *supra*). Portions of FAST-1 or Smad4 cloned into a GAL4 DNA binding domain fusion vector (pGBT9) were tested for their ability to

15   interact with various Smad-GAL4 activator domain (pGAD424) fusions.

Methods

Interaction trap constructs: Truncated derivatives of FAST-I and Smad genes were cloned into the shuttle/expression vectors pGBT9 and pGAD424 (or pGAD10) (Bartel et al., *Using the two-hybrid system to detect protein- protein*

20   *interactions*, p153-179. *In* D. Hartley (ed.), Cellular Interactions in Development: A Practical Approach, Oxford Press, Oxford). A fusion of the GAL4-DNA-binding domain in the pGBT9 vector with each FAST-1 truncation derivative was generated, as was a fusion of the GAL4 activation domain in the

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pGAD424/pGAD10 vector with each Smad.

Specifically, FAST-1-GAL4 DNA-binding domain fusion proteins in the pGBT9 vector included the following regions of FAST-1: (1) FAST-1 N-domain and C-domain (aa61 to aa516); (2) FAST-1 C-terminus (aa366 to aa518); (3) FAST-1 Δ207-453 (aa61 to aa515, with 6 Myc epitope tags at the junction with the GAL4-DNA-binding domain, with aa207 to aa453 deleted); (4) FAST-1 Δ366-407 (aa61 to aa515, with aa366 to aa407 deleted); (5) FAST-1 Δ366-439 (aa61 to aa515, with aa366 to aa439 deleted); (6) FAST-I forkhead domain (aa56 to aa365).

Smad-GAL4 activation domain fusion proteins in the pGAD424 or pGAD10 vector were generated that included the following Smad regions: (1) *Xenopus* Smad2 MH2 domain (aa248 to aa467); (2) human Smad1 MH2 domain (aa249 to aa465); (3) full-length mouse Smad4 (aa1 to aa548); (4) mouse Smad4 MH2 domain (aa306 to aa548). In addition, full-length Smad4 (aa1 to aa548) and the MH2 domain of Smad4 (aa306 to aa548) were cloned into the pGBT9 vector.

Transformation and testing of yeast with two-hybrid clones: Yeast transformations, colony lift filter assays were carried out according to the MATCHMAKER Two-Hybrid System protocol (Clontech Laboratories, Inc., Palo Alto, CA). For the filter assay, colony color was periodically observed during a 5-6 hour incubation at 30°C following initial exposure of permeabilized yeast to the Z buffer/X-gal solution. The liquid culture beta-galactosidase assay was performed according to the MATCHMAKER Two-Hybrid System protocol (Clontech Laboratories, Inc., Palo Alto, CA). O-nitrophenyl beta-D-galactopyranoside (ONPG) was used as a substrate in this assay. Beta-galactosidase units corresponding to each sample were calculated using the

following equation: Beta galactosidase units = $(1000 \times OD_{420}) \div (tV \times OD_{600})$ where: t= elapsed time (in minutes) of incubation, V= 0.1 ml X concentration factor of 5, OD_{600}= Absorbance at 600 nm of 300 microliters of Z buffer-washed and resuspended culture.

5   Positive results were measured either as the development of blue color on X-Gal filter lifts of colonies expressing both activator and DNA binding domain constructs relative to colonies expressing each construct alone, or as a ratio of β-galactosidase activity in liquid cultures expressing activator and DNA binding domain constructs relative to colonies containing the DNA binding domain

10   construct alone.

Results

          As shown in Table 1, the C-terminal third of FAST-1, to which the Smad2 co-immunoprecipitation function of FAST-1 mapped (FAST-1 366-518), interacted strongly with the MH2 domain of Smad2, whereas the winged helix

15   domain region (FAST-1 56-365), did not. The Smad2 MH2 domain, and Smad4 itself (in pGAD424, the activator domain construct) interacted with Smad4 when Smad4 was expressed in pGBT9 (DNA binding domain construct), confirming that the activator domain-Smad2 and -Smad4 fusion proteins were expressed, and that these proteins physically interact within the yeast assay. In contrast, the

20   C-terminus of FAST-1 did not interact detectably with the MH2 domain of Smad1, confirming the specificity of its interaction with the Smad2 MH2 domain. Nor did the FAST-1 C-terminus interact detectably with the MH2 domain of Smad4.

          Additional N-terminal deletions of the C-terminal third of FAST-1 which allowed us to distinguish regions necessary for ARF/ARE complex

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formation and Smad4 association from those necessary for Smad2
co-immunoprecipitation (i.e., FAST-1Δ366-407), showed that the region of FAST-
1 necessary for its interaction with Smad2 in yeast was similar to the FAST-1
region necessary for FAST-1/Smad2 co-immunoprecipitation. Although our
5   results from the yeast interaction trap assay the possibility that additional proteins
enhance Smad4/FAST-1 interactions (for example, yeast lack homologues for the
activin signalling pathway), our results indicate that activin signalling is not a
prerequisite for Smad2/FAST-1 interaction.

Table 1

Interactions of Smads with Themselves and FAST-1

Bait Construct	Interactor Construct	Color Intensity (Filters)
FAST-I (aa61-518)	Smad2 (MH2)	+
	Smad1 (MH2)	-
	Smad4 (Full Length)	-
	Smad4 (MH2)	-
FAST-1 (aa366-518)	Smad2 (MH2)	+
	Smad1 (MH2)	-
	Smad4 (Full Length)	-
	Smad4 (MH2)	-
FAST-I (aa56-365: Δ366-518)	Smad2 (MH2)	-
	Smad1 (MH2)	-
FAST-I (aa61-515: Δ366-407)	Smad2 (MH2)	+
	Smad1 (MH2)	-
FAST-I (aa61-515:Δ366-439)	Smad2 (MH2)	+
	Smad1 (MH2)	-
FAST-I (aa61-515: Δ207-453)	Smad2 (MH2)	+
	Smad1 (MH2)	-
Smad4 (FL)	Smad2 (MH2)	+
	Smad1 (MH2)	+
	Smad4 (Full Length)	+
Smad2 (MH2)	Smad4 (MH2)	+

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EXAMPLE VIII

Overexpressed FAST-1 SID domains blocks formation of the ARF

The identification of a domain in FAST-1 that is necessary for interaction with Smads raised the possibility of using this domain to competitively

5 inhibit activin signalling. Embryos were injected with mRNA encoding amino acids 366-518 of FAST-1 (the C-terminal third of FAST-1, containing the SID) and tested for their ability to respond to activin signals. We first asked whether the FAST-1 SID could inhibit activin-dependent ARF/ARE complex formation.

Methods

10 Two ng of mRNA encoding FAST-1 amino acids 366 to 518, plus or minus mRNA encoding activin, was co-injected into both blastomeres of two-cell *Xenopus laevis* embryos by the method described in Example I. Stage 9 embryos were harvested and tested for ARF/ARE complex formation by EMSA as described in Example I.

15 *Results*

Control embryos (Fig. 7, lanes 1, 2) and embryos injected with mRNA encoding FAST-1 366-518 (Fig. 7, lanes 3, 4) plus (Fig. 7, lanes 2, 3) or minus (Fig. 7, lanes 1, 4) activin stimulation were harvested at Stage 9 and lysates were tested for ARF/ARE complex formation by EMSA. As shown in Fig. 7,

20 overexpression of the Smad Interaction Domain of FAST-1 inhibited the formation of ARF/ARE complexes.

EXAMPLE IX

Overexpression of FAST-1 SID Blocks Brachyury Induction and Animal Cap Induction by Activin

The activin/TGFβ superfamily induces mesoderm formation in early
5   *Xenopus* embryos. Induction of mesoderm may be inferred by the detection of brachyury, a marker of early mesoderm, and by animal cap elongation.

We tested the effect of FAST SID overexpression upon activin-induced brachyury expression, and upon activin-induced animal cap elongation.

Methods

10   *Animal cap assays and RT-PCR*: Two ng of mRNA encoding the FAST-1 SID (FAST-1 amino acids 366 to 518) and/or 150 pg Smad2 RNA were microinjected into both blastomeres of two-cell *Xenopus laevis* embryos by the method described in Example I. Animal caps were cut from Stage 8-9 blastulae and cultured in 0.7 X MMR containing 0.1% gelatin, 100 μg/ml BSA, 250 μg/ml Gentamicin
15   (GIBCO BRL), and 200 pM purified recombinant activin (Ajinomoto, Inc.), or 100 ng/ml human recombinant bFGF (GIBCO BRL), either until control embryos reached Stage 10.5 (for RT-PCR), or until control embryos reached Stage 23/24 (for animal cap elongation photography). Staging of embryos was done according to Nieuwkoop and Faber, *supra*.

20   Total RNA was extracted from embryos and animal caps at Stage 10.5, and RT-PCR was performed as previously described (LaBonne and Whitman, Development 120: 463-472, 1994), using 20 amplification cycles for EF-1α and 25 for Xbra.

Results

Animals caps from embryos expressing FAST-1 SID (Fig. 8A, lanes 6-8, 10, 11) in the absence (Fig. 8A, lanes 1-8) or presence (Fig. 8A, lanes 9-11) of overexpressed Smad2 were tested for the induction of the pan-mesodermal marker

5   brachyury (Xbra). Fig. 8A shows an agarose gel containing electrophoretically resolved RT-PCR products from RNA extracted from animal caps cut at Stage 8-9, treated with activin or FGF, and harvested for RNA at Stage 10.5. The ubiquitously-expressed marker EF1α was also RT-PCR-amplified within each reaction, as an internal control for quantitation of brachyury PCR products. The

10   symbols "+F" and "-F" denote samples from embryos that were injected with · Smad2 mRNA plus or minus mRNA encoding the FAST-1 SID (FAST-1 aa366-518).

Activin-dependent induction of brachyury was inhibited by overexpressed FAST-1 SID. Moreover, inhibition was specific for the

15   activin/TGF-β signalling pathway, since induction of brachyury by bFGF was unaffected by the FAST-1 SID (Fig. 8A). Activin-dependent induction of the endo-mesodermal marker Mix.1 also was inhibited by the FAST-1 SID, as was induction of mesodermal markers by Smad2. However, overexpression of Smad2 partially restored activin-dependent gene expression in the presence of the FAST-1

20   SID. These observations suggest that inhibition of mesoderm-specific gene expression is due to the sequestration of Smads by the FAST-1 SID.

Fig. 8B shows a series of photographs of animal caps from control and experimentally-manipulated *Xenopus* embryos. Panel A shows unstimulated embryos; Panel B shows activin-stimulated embryos; Panel C shows activin-

25   stimulated/ FAST-1 SID-microinjected embryos; Panel D shows FGF-stimulated

embryos; Panel E shows FGF-stimulated/ FAST-1 SID-microinjected embryos; and Panel F shows unstimulated/ FAST-1 SID-microinjected embryos.

Activin-dependent elongation of animal caps, a marker of mesoderm induction, was inhibited by overexpressed FAST-1 SID (Fig. 8B, panel C). In
5   contrast, like bFGF-induced brachyury expression, bFGF-dependent animal cap elongation was not inhibited by overexpressed FAST-1 SID (Fig. 8B, panels C and E). These results indicate that FAST-1 SID specifically inhibits the activin/TGF-β signalling pathway.

EXAMPLE X

10   Specific binding of FAST-1 and Smad2 domains *in vitro*

The limitations of the yeast interaction trap method for monitoring protein-protein interactions (e.g., the difficulty of delivering compounds into yeast, and other non-specific effects due to yeast biology), made it desirable to develop an alternative method without such limitations. Hence, we developed an *in vitro*
15   method to detect interactions between the FAST-1 Smad Interaction Domain (SID) and Smad2 MH2 domain. This method allows the detection of inhibitors of activin/TGFβ superfamily signalling.

Methods

The SID of FAST-1 was fused to a GST tag, expressed in *E. coli*, and
20   isolated by binding to glutathione sepharose as described in an earlier section. As a control, GST was expressed and purified in parallel. Myc-tagged Smad1 or Smad2 MH2 domains were expressed in *Xenopus laevis* embryos by mRNA injection; Stage 9 embryos were lysed and assayed for MH2 domain expression

level by Western blot analysis using anti-Myc antibodies (lane 1, uninjected; lane 2, injected with Myc-Smad1 MH2; lane 3, injected with Myc-Smad2 MH2). Lysates from Stage 9 embryos expressing Myc epitope-tagged Smad1 (lanes 4 and 6) or Smad2 MH2 (lanes 5 and 7) domains were also incubated with GST-control

5   (lanes 4 and 5) or GST-FAST-1 SID (lanes 6 and 7) fusion protein. Protein complexes were precipitated by binding to glutathione-coated beads and precipitated proteins were resolved by Laemmli gel electrophoresis and subjected to Western blot analysis with anti-Myc antibodies.

10   *Results*

Fig. 9 shows a Western blot analysis, using anti-Myc antibodies, of samples from uninjected embryos (lane 1), embryos expressing Myc-Smad1 MH2 domain (lanes 2, 4, 6,) and embryos expressing Myc-Smad2 MH2 domain (lanes 3, 5, 7). Lanes 1, 2, and 3 are whole lysates, lanes 4 and 5 are immunoprecipitates

15   from lysates incubated with GST control protein, and lanes 6 and 7 are immunoprecipitates from lysates incubated with GST-FAST-1 SID. Fig. 9 shows that the FAST-1 SID specifically binds the Smad2 MH2 domain, but not the Smad1 MH2 domain (lane 7). That fact that these results, originally detected using the yeast interaction trap assay described in Example VII, may also be

20   observed in our *in vitro* assay, confirms the validity of this approach for monitoring specific FAST-1/Smad2 interactions.

EXAMPLE XI

Identification of human and mouse homologues of FAST-1

Since TGF-β superfamily signalling affects the development of a wide

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variety of organisms, we isolated cDNAs encoding mammalian homologues of *Xenopus* FAST-1.

We searched publicly available sequence databases for sequences with identity to the amino acid sequence of full length *Xenopus* FAST-1 (Chen et al., *supra*), and

5   for sequences with identity to amino acids 380 to 506 of FAST-1 (corresponding to the FAST-1 SID).

Our search for sequences with identity to full length FAST-1 identified no candidate FAST-1 homologues. However, by using the amino acid sequences corresponding to the FAST-1 SID as a probe to screen sequence listing databases,

10 we identified one sequence in the TIGR Human Gene Index (TIGR clone ID No. 64997; clones in the TIGR index are commercially available) with identity to the FAST-1 SID. The Genbank accession number of the human FAST-1 SID partial sequence is AA218611.

The human FAST1 SID clone had an insert size of approximately 300

15 base pairs, corresponding to a 100 amino acid long polypeptide. A cDNA clone encoding full-length human FAST-1 was isolated by screening a human cDNA library, by standard techniques, using the fragment encoding the human FAST-1 SID as a probe.

Degenerate primers were designed that correspond to regions conserved

20 between human and *Xenopus* FAST-1. The primers were used in PCR reactions that contained cDNA from mouse embryonic stem cells as a template. A partial cDNA encoding mouse FAST-1 was obtained, which was used to screen mouse cDNA and genomic libraries to obtain the full-length mouse FAST-1 sequence.

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EXAMPLE XII

FAST-1-like activity by the human FAST-1 homologue

We tested the candidate human homologue of FAST-1 for the ability to co-immunoprecipitate with Smad2.

5   *Methods*

The 300 base pair insert encoding the human FAST-1 SID was tagged with the Myc epitope by subcloning the insert into the pCS2(+)MT vector.

Myc-tagged human FAST-1 was co-expressed with GST-tagged Smad2 in *Xenopus* embryos in the presence or absence of activin stimulation. The

10   microinjected embryos were then lysed and immunoprecipitated with anti-GST antibody, followed by blotting with anti-Myc antibody, as described in Example IV.

Results

The human Myc-tagged FAST-1 SID co-immunoprecipitates with GST-

15   tagged Smad2, indicating that human FAST-1, like *Xenopus* FAST-1, is able to associate with Smad2. Also like *Xenopus* FAST-1, the human FAST-1 SID co-immuno-precipitates Smad4 in an activin-dependent-manner.

An amino acid sequence alignment of human, mouse, and *Xenopus* FAST-1 is shown in Fig. 10. Regions of FAST-1 polypeptides having identical

20   amino acids are boxed. The human and mouse FAST-1 SIDs are comprised, at maximum, of human FAST-1 amino acids 234-365, and mouse FAST-1 amino acids 309-398.

Other Embodiments

All publications and patent applications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication or patent application was specifically and individually indicated to be
5   incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention
10   and including such departures from the present disclosure come within known or customary practice within the art to which the invention pertains and may be applied to the essential features hereinbefore set forth.

What is claimed is: